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Algal-bacterial cooperation improves algal photolysis-mediated hydrogen production

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ABSTRACT

In this study, bacterium *Pseudomonas* sp. strain D was proved to be the main partner assisting *Chlamydomonas reinhardtii* in improving photolysis-mediated H_2 production and a good partner for promoting H_2 production by the green algae *Chlorella* and *Scenedesmus*. In strain D partnered algal-bacterial co-culture, the relative O_2 content in the headspace plus the dissolved oxygen in the culture medium were rapidly consumed by bacterial growth, resulting in a completely anaerobic environment that proved suitable for the activation of algal hydrogenase. Moreover, algal-bacterial cooperation was able to slow the reduction of chlorophyll, enhance starch accumulation, and maintain protein content, which are the potential factors whose control provides an opportunity for improving algal H_2 production. This study systematically analyzed the main pathway responsible for H_2 production by algal-bacterial cooperation and the potential mechanisms for improvement, and proposed an efficient and durable algal-bacterial cooperation system for improving photolysis-mediated H_2 production by green algae.

1. Introduction

Global warming has become an increasingly serious problem due to the anthropogenic emission of large quantities of greenhouse gases. The increasing demand for carbon emission reduction makes H_2 a more attractive fuel, since it is a clean and high energy-density fuel (Oey et al., 2016). Renewable H_2 production from algal photosynthesis has a great potential to become a next-generation biofuel because algae, (1) have higher productivity, (2) can be grown on non-arable land and, (3) have high solar energy conversion efficiency (Rashid et al., 2013; Oey et al., 2016). Green alga *Chlamydomonas reinhardtii* is a model algal species that has been used for studying photolysis-mediated H_2 production, because of its high hydrogenase activity, available genetic background and ease of cultivation (Melis, 2007; Oey et al., 2016). In addition to *Chlamydomonas*, several other green algae genera, including *Chlorella*, *Scenedesmus* and *Platymonas,* have also been reported to produce H_2 (Rashid et al., 2011; Chader et al., 2011; Zhang et al., 2012; Marquez-Reyes et al., 2015).

The H_2 producing activity in green algae is predominantly due [FeFe] hydrogenase, which has a unique active center with an output activity about 10–100-fold higher than [NiFe] hydrogenase, which occurs mainly in cyanobacterial species (Batyrova and Hallenbeck, 2017; Khetkorn et al., 2017). However, [FeFe] hydrogenase is highly O_2 sensitive. This sensitivity was documented to be the main bottleneck for algal photolysis H_2 production. Many solutions have been devised to reduce O_2 level to overcome this problem with algal H_2 production; for example, sulfur deprivation is currently a widely used strategy, because it partially inactivates PSII activity, thus maintaining photolysis O_2 production below the rate of mitochondrial respiration, which in turn results in the required anaerobic condition for H_2 production (Melis et al., 2010; Eroglu and Melis, 2016; Oey et al., 2016; Batyrova and Hallenbeck, 2017). Besides nutrient deprivation, the addition of O_2 scavengers, including chromous chloride, dithionite, and cysteine, or O_2 purge by rinsing the culture atmosphere with inert gases have also been tested to keep the atmosphere around algae cultures free of O_2 (Marquez-Reyes et al., 2015; Batyrova and Hallenbeck, 2017).

Furthermore, co-cultivation of algae and bacteria (such as *Azotobacter, Bradyrhizobium, Pseudomonas,* and *Escherichia*) has also been reported as a useful method to achieve anaerobiosis by the respiration of bacterial partners (Wu et al., 2012; Li et al., 2013; Lakatos et al., 2016; Xu et al., 2016, Xu et al., 2017). The cooperation between algal and bacterial cells in co-culture exhibits a co-metabolism pattern: the bacteria removes the O_2 sensitivity from algal hydrogenase, while the alga supplies O_2 and organics for bacterial growth. However, there

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was very little research work carried concerning deeper reasons for the improvement of algal-bacterial cooperated hydrogen production after the removal of O_2 sensitivity. In this study, we aimed to identify bacterial cooperated effect on algal photolysis-mediated H_2 production, and systematically analyzed the potential mechanisms (such as slowing chlorophyll reduction, enhancing starch accumulation, and maintaining protein content) that mainly responsible for the improvement of H_2 production.

2. Materials and methods

2.1. Algal and bacterial strains

Algal strains *Chlamydomonas reinhardtii* FACHB-265, *Chlorella protothecoides* FACHB-3, *Chlorella pyrenoidosa* FACHB-5 and *Scenedesmus obliquus* FACHB-416 were obtained from the FACHB collection (http://algae.ihb.ac.cn/), Institute of Hydrobiology, Chinese Academy of Sciences. Algae *Chlorella* sp. WFY and *Scenedesmus* sp. WFY were isolated from the Pearl River water and maintained in our laboratory. Algal cells were purified according to an antibiotic procedure (Lin et al., 2017). Algal cultivation was performed in 250-mL glass flasks filled up with 100 mL of TAP media. Phototrophic growth was maintained at 30 °C in an illumination incubator (ZQLY-180, Zhichu Instruments Co., Ltd, Shanghai, China) under a light density of 50 μmol $m^{-2} s^{-1}$, coupled to a 12/12-h light/dark photoperiod.

Bacterial strains *Escherichia coli* DH5α and *Bacillus subtilis* WB800 were stored in our laboratory and maintained on agar slants containing Luria-Bertani medium. Bacterial partners in the contaminated algal culture were isolated according to the following procedure: bacterial flora in the algal culture was obtained by extruding through a 0.8 μm membrane and spread on agar plates containing TAP medium (consisted of 20 mM Tris base, 7 mM NH_4Cl, 0.83 mM $MgSO_4$, 0.45 mM $CaCl_2$, 1.05 mM KH_2PO_4, 1.65 mM K_2HPO_4, 134 μM Na_2EDTA, 136 μM $ZnCl_2$, 184 μM H_3BO_3, 40 μM $MnCl_2$, 32.9 μM $FeCl_2$, 12.3 μM $CoCl_2$, 10 μM $CuCl_2$, 0.928 μM $(NH_4)_2Mo_7O2_4$, and 1 mL L^{-1}Acetic acid) after a serial dilution; bacterial colonies with clearly different phenotypes were picked and streaked on fresh agar plates for purification. Bacterial strains and isolates used for co-cultivation with algae were harvested from agar plates or slants and grown on liquid TAP medium at 30 °C, in the dark.

2.2. Co-cultivation of algae and bacteria for H_2 production

Algal and bacterial cells were harvested by the centrifugation at 1500 and 8000*g*, respectively. Cell pellets were washed three times in TAP-S media (to prepare sulfur-deprived TAP medium, $MgSO_4$ was replaced by $MgCl_2$) before inoculation. Algal and bacterial stock solutions were transferred into 100 mL Hypo-Vial bottles, resulting in a final volume of 50 mL TAP-S culture media containing 1.0×10^7 algal cells and 1.0×10^7 bacterial cells per milliliter of culture media. The bottles were sealed with butyl rubber stoppers and aluminum caps and continuously incubated at 30 °C under 50 μmol $m^{-2} s^{-1}$ light density. Pure algal and bacterial cultures with same cell number were used as controls.

2.3. Identification of bacterial partners in contaminated algal cultures

The complete genomic DNA of bacterial isolates were extracted and purified using a TaKaRa MiniBEST Bacteria Genomic DNA Extraction Kit (TaKaRa, Dalian, China). Bacterial 16S rRNA gene was obtained by PCR amplification using universal primer pair 27F/1492R (27F: 5′-AGAGTTTGATCCTGGCTCAG-3′; 1492R: 5′-TACCTTGTTACGACTT-3′) (Frank et al., 2008). PCR amplification was performed in a 25 μl reaction mixture containing 1 μl of DNA template, 12.5 μl of PCR mix (TIANGEN, China), and 1 μl of forward and reverse primers (10 μM). The PCR products were purified using the TaKaRa Agarose Gel DNA Purification Kit (TaKaRa, Dalian, China) and sequenced on a MiSeq 300 sequencer (Illumina, San Diego, USA) by IGE Biotechnology (Guangzhou, China). Taxonomic classification of bacterial 16S rRNA gene was analyzed using the Nucleotide-nucleotide BLAST (blastn) database. A phylogenetic tree was constructed by the MEGA program using neighbor-joining algorithms (Tamura et al., 2011).

2.4. Analytical methods

Algal cell number was measured by cell counting performed with a hemocytometer and bacterial cell number was determined on TAP plates using serial dilutions.

Chlorophyll content in algal cells was determined as previously reported (Luo et al., 2013). Briefly, algal cells from pure cultures were collected by centrifugation at 1500*g*, while algal cells from co-cultures were obtained by retention on a 0.8 μm membrane; pellets were re-suspended in 95% (v/v) ethanol and incubated at 75 °C for 5 min; the supernatant was collected by centrifugation at 10,000*g* for 5 min and used for absorbance determination at 649, 664 and 750 nm with a spectrophotometer (Unico UV2802S, Shanghai, China). Chlorophyll content was calculated according to the following equation: $Chl = 5.24 \times (A_{664} - A_{750}) + 22.24 \times (A_{649} - A_{750})$.

Starch determination was performed by a method previously reported (Fouchard et al., 2005), after a slight modification. Briefly, the collected algal cells were suspended in 95% (v/v) ethanol for chlorophyll extraction and centrifuged again. The pellets were rinsed and resuspended with acetate buffer (100 mM, pH 4.5) and autoclaved for 30 min in boiling water for starch solubilization. Starch was then measured by using a Megazyme starch assay kit (Megazyme, Ireland) following manufacturer instructions.

Before protein determination, collected algal cells were suspended in 95% (v/v) ethanol and incubated at 75 °C for chlorophyll extraction; pellets were obtained by centrifugation at 10,000*g* for 5 min, rinsed with distilled water and resuspended in 1 M NaOH solution; the supernatant was incubated for 30 min in boiling water and then used to determine protein concentration according to Lowry (Lowry et al., 1951).

The H_2 and O_2 levels in the headspace of the Hypo-Vial bottles were measured by gas chromatography. A GC9790 Plus gas chromatograph (Fuli instruments, China) equipped with a thermal conductivity detector (TCD) and a 5 Å molecular sieve column (2 m × 1/8 mm) was used. Argon was used as carrier and reference gas. Temperatures of the injector, TCD and column were kept at 70 °C, 100 °C and 60 °C, respectively; flow rate of the column was 20 mL min^{-1} and injection volume was 1.0 mL. H_2 and O_2 concentrations were calculated by the external standard method.

Dissolved oxygen concentration in the liquid phase of the cultures was measured using a ST400D optic oxygen meter (OHAUS, USA).

2.5. Statistical analysis

Each experiment was carried out in triplicate. Mean and standard deviation values were analyzed using one-way analysis of variance (ANOVA). Significance of difference was determined as $P < .05$.

3. Results and discussion

3.1. Photolysis-mediated H_2 production by pure and bacteria-contaminated algal culture

H_2 production was performed by *C. reinhardtii* cells in a nonsterile TAP-S medium which became contaminated with bacteria from the laboratory environment. Contaminating bacterial flora was collected by differential centrifugation and inoculated into pure algal cultures for H_2 production. As can be seen in Fig. 1a, bacterial flora showed no H_2 accumulation during cultivation, while pure algal culture accumulated